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### **Increased CXCL10 expression in MS MSCs and monocytes is unaffected by AHSCT**

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## RESEARCH ARTICLE

# Increased CXCL10 expression in MS MSCs and monocytes is unaffected by AHSCT

Elena Bonechi<sup>1,a</sup>, Alessandra Aldinucci<sup>1,a</sup>, Benedetta Mazzanti<sup>2</sup>, Massimo di Gioia<sup>2</sup>, Anna Maria Repice<sup>1</sup>, Cinzia Manuelli<sup>3</sup>, Riccardo Saccardi<sup>2</sup>, Luca Massacesi<sup>1</sup> & Clara Ballerini<sup>1</sup>

<sup>1</sup>Dept. NEUROFARBA, University of Florence, Florence, Italy

<sup>2</sup>Hematologic Unit, Careggi University Hospital, Florence, Italy

<sup>3</sup>Dept. DMSC, University of Florence, Florence, Italy

## Correspondence

Clara Ballerini, Dept NEUROFARBA, Viale Pieraccini, 6, 50139 Florence, Italy.

Tel: +39 0554271377;

Fax: +39 0554271380;

E-mail: clara.ballerini@unifi.it

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<sup>a</sup>These authors equally contributed to the work.

## Abstract

**Objective:** To confirm CXCL10 over production in bone marrow mesenchymal stem cells (MSCs) and circulating monocytes isolated from multiple sclerosis patients (MS) and identify predate cell molecular signature; to extend this analysis after autologous hematopoietic stem cell transplantation (AHSCT) to test if therapy has modifying effects on MSCs and circulating monocytes. **Methods:** MSCs and monocytes were isolated from 19 MS patients who undergone AHSCT before and seven of them at least 3 years after transplant. CXCL10 production was detected after LPS/IFN- $\gamma$  stimulation. TLR4 signaling pathways were investigated by means of transcription factors phosphorylation/activation level. RT-PCR of activated transcription factors was performed to quantify their expression. All experiments were conducted in parallel with 24 matched healthy donors (HD). **Results:** CXCL10 expression was significantly increased in both peripheral circulating monocytes and BM MSCs compared to HD. We showed that CXCL10 production is determined by an altered signaling pathway downstream TLR4, with the involvement of STAT-1, NF- $\kappa$ B, p38, JNK, and CREB. All upregulated transcription factors are more phosphorylated in MS patient sample. These features are not modified after AHSCT. **Interpretation:** We demonstrated that in MS two different cell lineages are characterized by significantly increased production of CXCL10, due to altered signaling pathways of innate immune reaction mediated by TLR4, probably associated with disease phenotype. This characteristic is not modified by AHSCT, suggesting that when T and B lymphocytes are reset, other possible components of MS pathology, such as CXCL10 over production, do not determine therapy outcome.

## Introduction

Chronic inflammatory diseases such as multiple sclerosis (MS) are mediated by the interaction between professional immune cells and tissue-resident cells. Together with many factors, the perpetuation of inflammatory cytokines and chemokines production may maintain inflammation with consequent impairment of organ tissue. The CXC motif chemokine 10 (CXCL10) is an inflammatory chemokine that may be produced by different cell types such as monocytes, dendritic cells (DC), endothelial and epithelial cells, and keratinocytes, in response to IFN $\gamma$ .<sup>1</sup> CXCL10 is involved in inflammatory

processes ongoing in RR and SP forms of MS and in a previous work, we showed that bone marrow (BM)-derived mesenchymal stem cells (MSCs) isolated from MS show increased production of CXCL10.<sup>2</sup> In this work, Mazzanti et al. suggested that BM, containing a reticulum of cells collectively referred to as stromal cells and MSCs which provide a supportive microenvironment for growth, differentiation, and proper function of the hematopoietic stem cells (HSCs), may be indicative of a more wide inflammatory status and may be involved in disease course and in the outcome of autologous hematopoietic stem cell transplantation (AHSCT) treatment. Although BM has not been widely investigated, several

authors reported the presence of increased CXCL10 in serum and cerebrospinal fluid (CSF) of MS, together with other inflammatory chemokines.<sup>3,4</sup> Recently, we observed in 50 MS CSF, lumbar puncture during acute phase of disease, that CXCL10 was overexpressed in all analyzed samples, and this was not true for other chemokines (e.g., CXCL13) or cytokines that were only sporadically present (e.g., IL-17, IFN $\gamma$  or TNF $\alpha$ ; C. Ballerini, pers comm 2013). Together, these findings suggest a role of CXCL10 in MS, such as potent chemoattractant for T lymphocytes, particularly Th1, NK, and monocytes, cells that participate of central nervous system (CNS) infiltration determining MS pathology. Here, we analyzed for the first time CXCL10 production in MS MSCs and peripheral blood (PB) monocytes before and after AHSCT. We found that in a sample of 19 MS patients refractory to conventional therapies, augmented CXCL10 production in two different cell lineages, BM MSCs, and blood circulating monocytes was a marker of disease and our data show that this was not due to an altered IFN $\gamma$  production and signaling, but depends on altered innate immunity TLR4-mediated signaling pathway. AHSCT has the capacity to almost completely suppress inflammation, can arrest the progression of disability in 50–70% of severe MS cases, is a powerful therapy for the malignant forms of MS, and at least with high intensity conditioning regimens, can induce a reset of the immunological clock for a period of time of at least 2 years inducing profound and long-lasting qualitative immunological changes.<sup>5,6</sup> CXCL10 is involved in many inflammatory diseases including MS and it has been suggested as therapeutic target,<sup>7,8</sup> here we investigated if CXCL10 was a target addressed by AHSCT therapy. In our study, we found that CXCL10-augmented production remains unaltered after treatment, indicating that the efficacy of transplantation therapy resides in lymphocytic cell reset and is not influenced by altered chemokine production in BM stromal cells and in circulating monocytes. Furthermore, our findings add important experience to the, generally speaking, stem cell therapy in autoimmune diseases investigating more in detail MS MSCs molecular signature and MS BM microenvironment.

## Methods

### Patients

A total of 19 MS patients (10RR/9SP) were enrolled for AHSCT at the Hematology Unit of Careggi University Hospital, Florence Italy; characteristics are presented in Table 1. AHSCT protocol was reported in a previous work.<sup>9</sup> Twenty-four healthy donors, matched for age and gender, were selected among orthopedic patients and/or BM donors. Local Ethical Committee approval # Prot.

467/11 was obtained for the study and informed consent was signed by all patients.

### Cells

BM-derived MSCs were isolated and characterized as previously reported,<sup>2</sup> the same for T lymphocytes. Monocytes were separated from frozen PB cells ( $10^6$  average cell number) by mean of positive CD14<sup>+</sup> microbeads selection (Miltenyi Biotec, Bologna, Italy) as reported by Aldinucci *et al.*<sup>10</sup> mixed lymphocyte reaction (MLR) and anti-CD3-CD28 stimulation of T cells and proliferative responses as reported in Mazzanti *et al.*<sup>2</sup>

### Soluble factors determination

CKs and chemokines production was evaluated in cell supernatants after 24 h of stimulation (LPS 1  $\mu$ g/mL; Sigma; Milano, Italy IFN $\gamma$  10 ng/mL; eBioscience San Diego CA USA) and in plasma by ELISA (Quantikine Human CXCL10 kit; R&D system; Minneapolis MN USA VeriKine Human IFN $\beta$  kit; PBL Interferon Source, Piscataway NJ USA) and Milliplex (Milliplex MAP kits #HCYTOMAG-60K, #HCVD1-67AK, #HBN1A-51K, #MPXHCYP2-62K-01; Merck Millipore Darmstadt Germany), following the manufacturer's instructions.

### Protein phosphorylation

MS MSCs and HD CREB, STAT-1, p38, JNK protein phosphorylation have been evaluated by Milliplex (Milliplex MAP Cell Signaling Buffer and Detection kit #48-602; Merck Millipore) following the manufacturer's instructions.

### Real-time PCR

MSCs and monocytes mRNA samples extracted and quantified as reported in Aldinucci *et al.*<sup>10</sup> were investigated by means of real-time PCR for IFN $\beta$ , NF- $\kappa$ B, I $\kappa$ B, STAT-1, and CXCL10 using an ABI Prism 7900HT Sequence Detection System (Applied Biosystem, Foster City CA USA). All PCR amplifications were performed on MicroAmp optical 96-well reaction plate with Taqman Universal Master Mix and using Assay on Demand (Applied Biosystem). Each assay was carried out in duplicate and included a no-template sample as negative control. RT negative samples were used to demonstrate that the signals obtained were RT dependent. Relative expression of mRNA levels was determined comparing experimental levels to a standard curve generated using serial dilutions of cDNA obtained from human PBMCs.  $\beta$ -actin expression levels were used as housekeeping gene for normalization.

**Table 1.** Patient characteristics.

Patients no.	Gender (F:M)	Age at AHSCT median (range)	Disease duration years median (range)	EDSS median (range)	Last therapy before AHSCT no. of patients/total no. of patients)					
					Cy	Mtx	Nb	Fg	IFN $\beta$	PE
Eleven RR-MS	8:3	28 (17–43)	10 (2–20)	6.0 (2–6.5)	5/11	2/11	2/11	1/11	0/11	1/11
Eight SP-MS	8:0	30 (19–53)	20 (11–33)	6.5 (4–6.5)	6/8	1/8	0/8	0/8	1/8	0/8

Cy, cyclophosphamide; Mtx, mitoxantrone; Nb, natalizumab; Fg, fingolimod; IFN $\beta$ , interferon  $\beta$ ; PE, plasma exchange.

## Statistics

Results were expressed as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using Student's *t*-test and Mann–Whitney test where appropriate. Statistical significance was for  $P < 0.05$ .

## Results

### MS and HD expression of CXCL10 in BM MSCs and circulating monocytes

MSCs isolated from MS patients produced increased amount of CXCL10 under LPS stimulation, compared to controls. Here, we tested MSCs isolated from 19 MS patients (Table 1) and 24 age-matched controls for CXCL10 production at three different passages after LPS stimulation (p2, p4, p6, Fig. 1A). We show that MS MSCs producing higher quantity of CXCL10 maintain this peculiarity during cell culture. In addition, we tested by RT-PCR CXCL10 mRNA production and again MS MSCs have significant ( $P < 0.05$ ) increased quantity of mRNA specific for the investigated chemokine (Fig. 1B). Among the tested cytokines (IL-1 $\alpha$ , $\beta$ ; IL-2-4-6-8-10-12p40-12p70-17-23, TNF $\alpha$ , OPN, IFN $\gamma$ , GM-CSF, MMP9, CXCL13, IFN $\beta$ ), we detected production of GM-CSF, IL-8, IL-6, and OPN; these factors were produced at the same extent in all samples (Fig. 1C).

Increased CXCL10 production after stimulation of TLR4 in our cohort of MS patients may be present in PB cells, that is, monocytes. In Figure 1D, we show that resting monocytes produced significant ( $P < 0.03$ ) higher amount of CXCL10 in MS sample compared with HD and the same was found upon LPS stimulation ( $P < 0.03$ ). Production of CXCL10 is inducible by interferons, therefore in the same samples we evaluated CXCL10 production after IFN $\gamma$  stimulation. We showed no differences in these experimental conditions (Fig. 1E), suggesting the exclusive involvement of TLR4 signaling pathway in altered CXCL10 production.

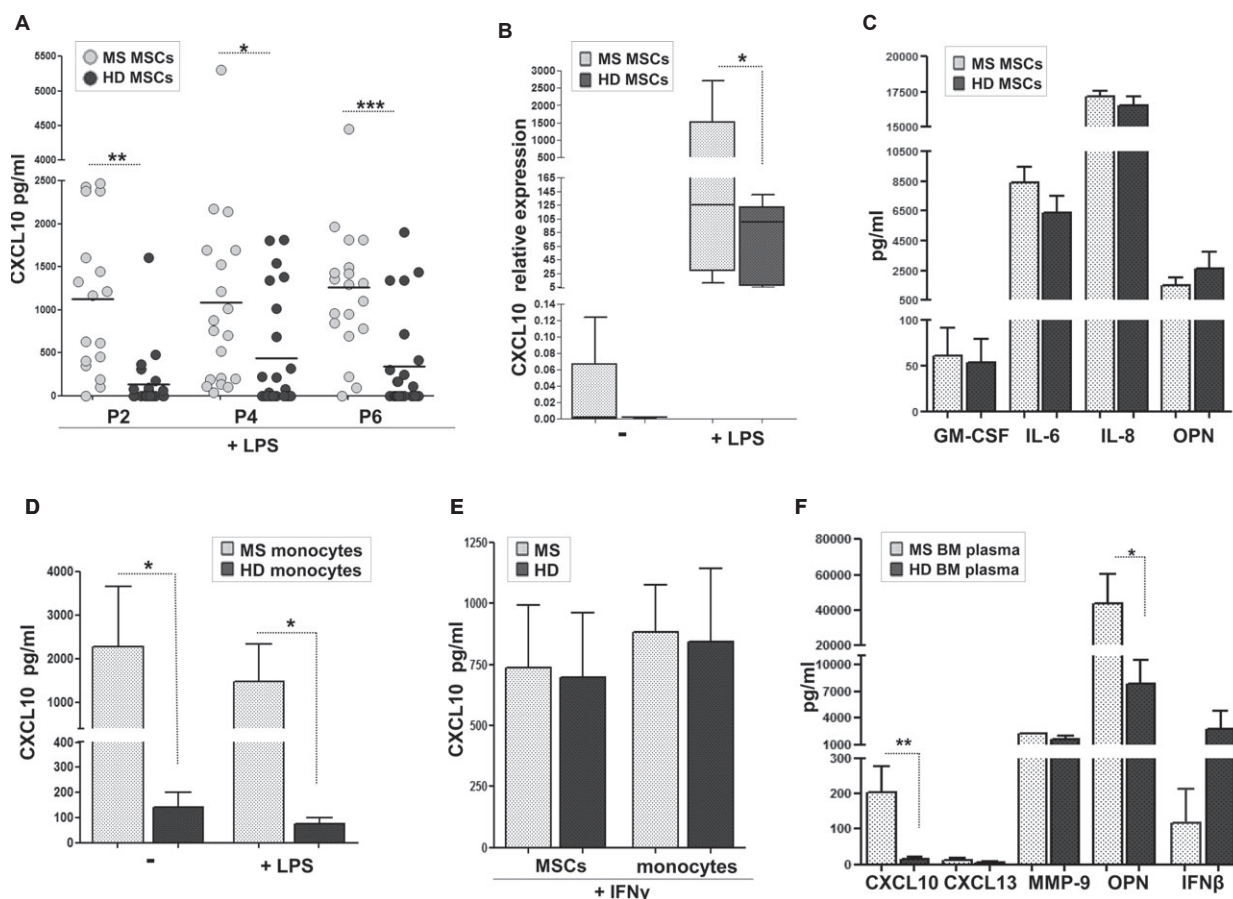
To gain insight into ex vivo cytokine production, we analyzed BM plasma from patients and controls for the

same soluble factors as we did in cell supernatants: in Figure 1F, we show that CXCL10 and OPN were present in a significant different quantity ( $P = 0.01$  and  $P = 0.04$ , respectively) representing a possible marker of MS BM.

Finally, we investigated if AHSCT treatment determines a difference in CXCL10 production in MSCs, BM plasma, and circulating monocytes. Seven MS patients underwent BM biopsy at a median follow-up of 4.5 years (range 3–7) from AHSCT (clinical data in Fig. 2A); all patients were monitored after the transplant and no clinical signs of flair were detected. One patient showed one Gd-enhancing area 7 years after the procedure. No immunosuppressive treatments were administered after the transplant. We showed that MS MSCs and monocytes preserved higher CXCL10 in vitro production after LPS stimulation (Fig. 2B and C), as well as CXCL10 amount is unmodified in BM plasma (Fig. 2D). We may conclude that monocytes/MSCs production of this fundamental T-cell homing inflammatory chemokine is a characteristic of our MS sample and it is not altered by AHSCT. In our sample, CXCL10 did not correlate with therapy outcome.

### MSCs TLR4 signal transduction protein analysis

To better understand which factors are involved in TLR4 stimulation and consequent CXCL10 production, we analyzed in MS and HD MSCs phosphorylation state of p38, CREB, JNK, ERK, and STAT-1. We tested five MS MSCs and five HD, on basal condition and upon 30 min (p-38, CREB, JNK, ERK) or 2 h (STAT-1) of LPS stimulation. MS MSCs expressed higher phosphorylation levels of p-38, CREB, and JNK after LPS stimulation compared with HD MSCs ( $P < 0.05$ ) (Fig. 3A). p38, JNK, CREB, and STAT-1 could be the signaling factors responsible for CXCL10 production in MS MSCs under LPS stimulation. On the other hand, MS and HD MSCs showed no change in the phosphorylation level of ERK (not shown), suggesting that ERK was not implicated in the signaling cascade responsible for CXCL10 production. Analysis of phosphorylation in samples post-AHSCT showed the same level of factor activation (Fig. 3B).



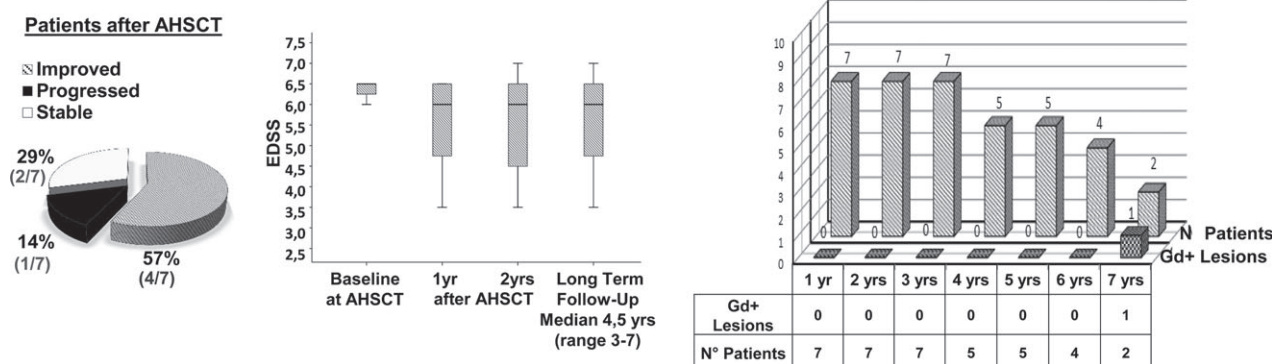
**Figure 1.** MSCs/monocytes CXCL10 production before AHSCT. (A) MSCs from 19 multiple sclerosis (MS) patients (gray circles) and 24 healthy donors (HD, black circles) were analyzed for CXCL10 production upon LPS stimulation at three in vitro passages, P2, P4, and P6. MSCs were plated in 24-well plates at the density of  $1 \times 10^5$  cells/mL, incubated for 24 h with LPS  $1 \mu\text{g/mL}$ . CXCL10 was measured on cell supernatants by ELISA. Each point in the graph corresponds to an independent MSC sample; horizontal bars represent CXCL10 mean value (pg/mL). Here, we show that MS MSCs produce higher level of CXCL10 with respect to controls at each tested passage. CXCL10 mean production is significantly higher in MS MSCs than HD MSCs at each passage (\*\* $P < 0.002$  at P2, \* $P < 0.05$  at P4, \*\*\* $P < 0.001$  at P6, nonparametric two-tailed  $t$ -test). (B) CXCL10 production was tested by real-time PCR on eight MS MSCs (gray Whiskers box plot) and eight HD (black Whiskers box plot) in basal condition (-) and upon stimulation with LPS for 4 h (+LPS). mRNA expression level is reported as ratio to  $\beta$ -actin. CXCL10 mean expression in MS MSCs is significantly higher than HD samples under stimulated condition (\* $P < 0.05$ , nonparametric two-tailed  $t$ -test). (C) All MS and HD MSCs included in the study were tested after LPS stimulation, for the production of the cytokines IL-1 $\alpha$ , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, TNF $\alpha$ , OPN, IFN $\gamma$ , GM-CSF, MMP9, and CXCL13 by Milliplex, and for IL-8, IFN $\beta$  production by ELISA. Here, we show that MSCs produce GM-CSF, IL-8, IL-6, and OPN, without any difference between MS and HD samples (mean values, expressed as pg/mL,  $\pm$ SEM are reported). (D) CXCL10 production was investigated in six MS and six HD samples of peripheral CD14<sup>+</sup> monocytes under basal condition (-) and upon LPS stimulation (+LPS) by Milliplex. Monocytes were plated in 24-well plates at the density of  $1 \times 10^6$  cells/mL and then incubated for 24 h with or without LPS  $1 \mu\text{g/mL}$ . Here, we show that MS monocytes produce higher level of CXCL10 than controls before and after LPS stimulation ( $P < 0.03$ , Mann-Whitney test). (E) In the same MSCs and monocytes samples analyzed as above, we evaluated CXCL10 production after IFN $\gamma$  stimulation (10 ng/mL for 24 h) by ELISA. All MSCs and monocytes produce CXCL10 in response to IFN $\gamma$ , without any difference between MS and HD. (F) BM plasma of seven MS patients and six HD was tested ex vivo for cytokines. The presence of IL-1 $\alpha$ , IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17, IL-23, TNF $\alpha$ , OPN, IFN $\gamma$ , GM-CSF, MMP9, and CXCL13 was evaluated by Milliplex; IL-8 and IFN $\beta$  were tested by ELISA. Among the tested cytokines, we found that BM plasma contains CXCL10, CXCL13, MMP-9, OPN, and IFN $\beta$ ; MS samples contain significantly higher amounts of CXCL10 (\*\* $P = 0.01$ , Mann-Whitney test) and OPN (\* $P = 0.04$ , Mann-Whitney test) than HD ones. HD, healthy donors; MS, multiple sclerosis; MSCs, mesenchymal stem cells.

### MSC/monocyte expression of TLR4 signaling factors

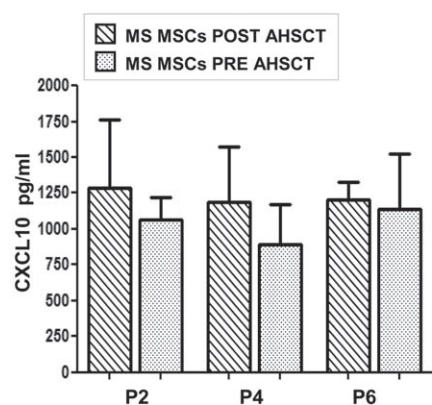
Transductional analyses indicated that TLR4 signaling cascade is altered in terms of activation. Level of mRNA

was quantified for p38, STAT-1, JNK, CREB, NF- $\kappa$ B, and I $\kappa$ B on basal conditions and LPS stimulation (4 h) by real-time PCR in MS and HD MSCs and monocytes. In patient cells, STAT-1, NF- $\kappa$ B, and I $\kappa$ B expression levels were significantly upregulated ( $P < 0.02$ ) after LPS stimu-

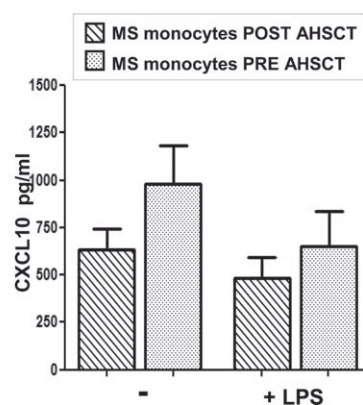
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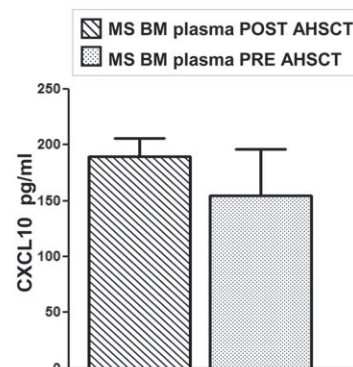
B



C



D



**Figure 2.** CXCL10 production after AHST. (A) Follow-up and clinical characteristics of seven of 19 MS patients after AHST. Left panel: at long-term follow-up, with a median of 4.5 years (range 3–7), patients had stable EDSS (29%), improved EDSS (57%) and only 1 (14%) progressed. Middle panel: whiskers box plots show EDSS modification according to the time from AHST. Right panel: no MRI activity was evidenced after AHST except for one patient who showed a new enhanced lesion 7 years after AHST, in absence of clinical relapse or clinical progression. (B) MSCs CXCL10 production after AHST. CXCL10 production was measured by ELISA in supernatants of MSCs isolated from seven patients before (pre) and after AHST (post) at three different passages, P2, P4, and P6, after LPS stimulation (24 h). MSCs POST AHST produce CXCL10 as MSCs PRE AHST. (C) CXCL10 production in peripheral monocytes isolated from four MS patients before and after AHST. CXCL10 production was tested (Milliplex) in basal condition (–) and upon stimulation with LPS for 24 h (+LPS). As shown by column bar graph (pg/mL mean  $\pm$  SEM), monocytes POST AHST produce CXCL10 at similar extent with respect to monocytes PRE AHST. MS, multiple sclerosis; MSCs, mesenchymal stem cells; AHST, autologous hematopoietic stem cell transplantation.

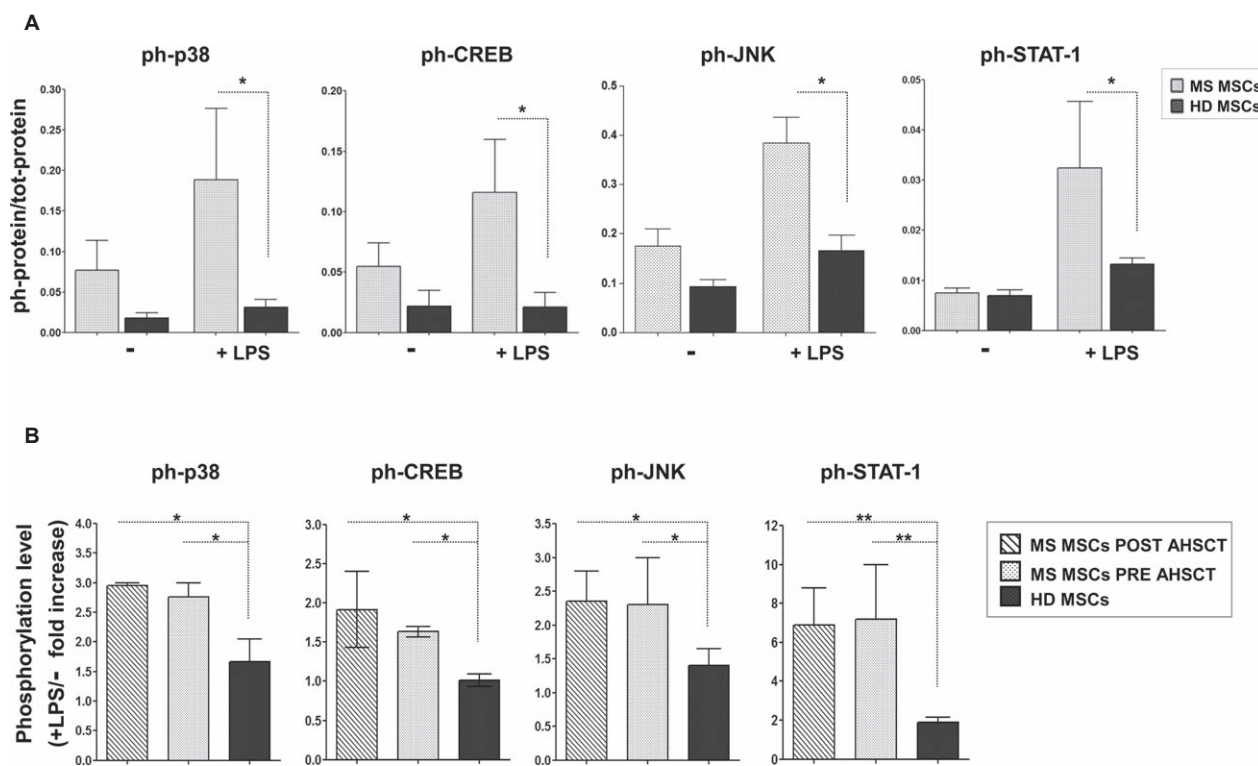
lation (Fig. 4A and B), whereas p38, JNK, and CREB not (not showed). Furthermore, we found a significant correlation (Fig. 4C and D) among STAT-1, NF- $\kappa$ B, and CXCL10 production ( $P < 0.01$ ). In Figure 4E, we showed that expression levels of analyzed factors remain unaltered post-AHST in MSCs and monocytes.

## Discussion

CXCL10, a chemokine with inflammatory and homing role, is increased in serum and CSF of MS patients indicating that MS pathophysiology is, in part, supported by CXCL10 production by different cell types.<sup>11</sup> Here, we

found that BM-derived MSCs and PB monocytes isolated from RR/SP MS produced significant increased amount of CXCL10 when stimulated through TLR4, independently on the RR/SP clinical course (data not shown). Of note, we measured an in vitro response in cultured cells that is in agreement with ex vivo results in BM plasma and strongly suggests that this might be a representation of in vivo situation. Indeed, innate immune response has been involved in MS pathogenic mechanisms in periphery and in CNS and it is believed that pathogen-associate molecular patterns (PAMPs) receptors have a role in determining MS inflammatory pattern and possibly influence clinical course as it happens in MS animal model.<sup>12,13</sup> The specific

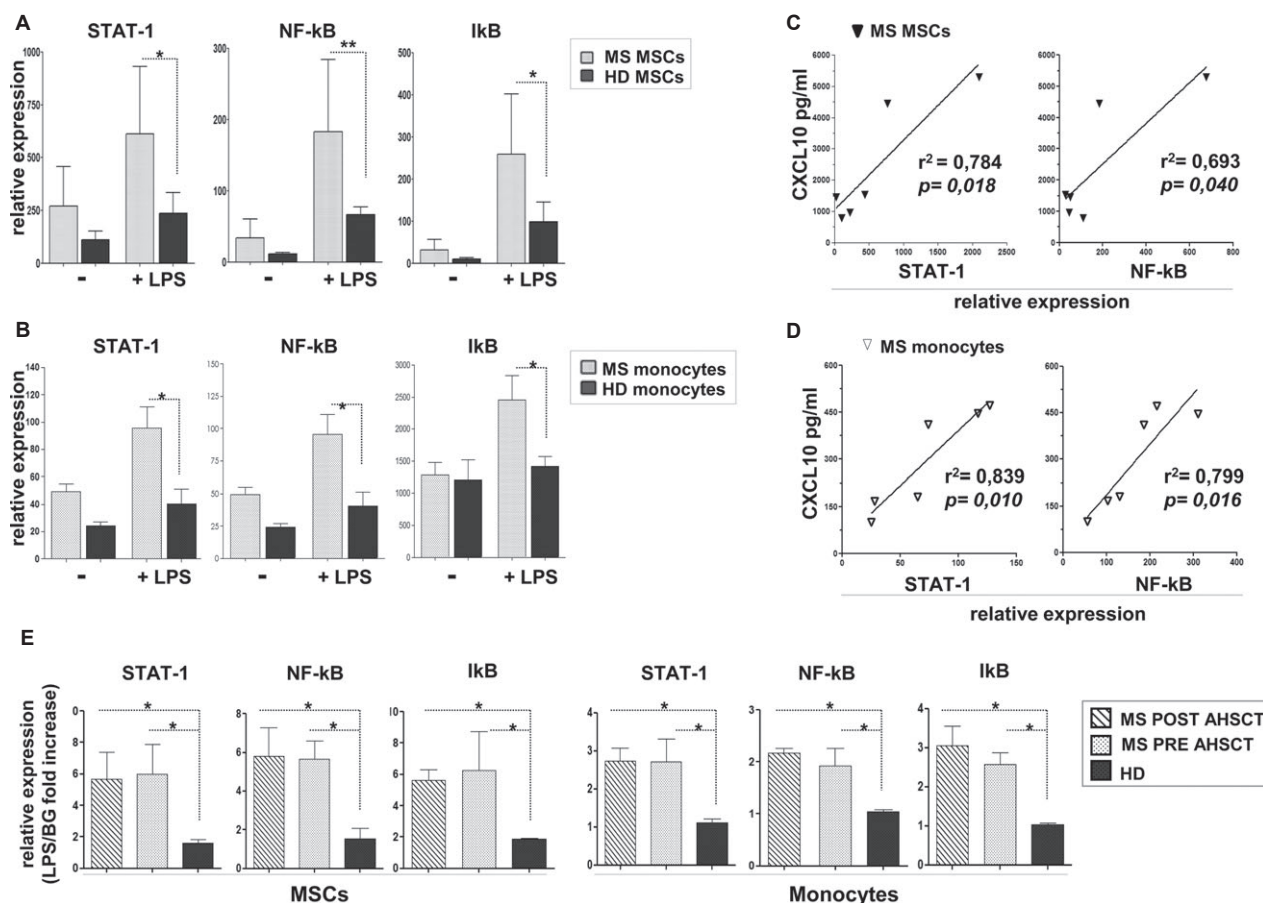




**Figure 3.** Signal transduction protein analysis. (A) The activation state of the signaling proteins p-38, CREB, JNK, and STAT-1 was investigated by Milliplex in five MS MSCs and five HD ones, on basal condition (–) and upon LPS stimulation (30 min for p-38, CREB and JNK, 2 h for STAT-1). For each analysis, 10  $\mu$ g of total extracted proteins was used. The graphs show the ratio between phosphorylated protein and total protein levels (ph protein/tot protein), expressed as mean value  $\pm$  SD. MS MSCs have higher level of ph p-38, ph CREB, ph JNK, and ph-STAT-1 than HD MSCs (\* $P$  < 0.05, Mann–Whitney test). (B) The activation state of p-38, CREB, JNK, and STAT-1 was also investigated in MS MSCs isolated after AHST (MS MSCs POST AHST) from the same patients analyzed in A) The phosphorylation level upon LPS stimulation is expressed as fold increase with respect to basal condition (ratio between ph protein/tot protein value in stimulated condition and ph protein/tot protein value in basal condition) and compared with pre-AHST (MS MSCs PRE AHST) and HD values (HD MSCs). Here, we show that after AHST MS MSCs preserve the same phosphorylation levels of p-38, CREB, JNK, and STAT-1 observed before AHST, and these are significantly increased compared with HD samples (\* $P$  < 0.04; \*\* $P$  < 0.001). HD, healthy donors; MSCs, mesenchymal stem cells; AHST, autologous hematopoietic stem cell transplantation; MS, multiple sclerosis; MSCs, mesenchymal stem cells.

involvement of TLR4 in our altered CXCL10 production is in agreement with our data on phosphorylation of molecules associated with TLR4 signaling pathway, augmented after LPS stimulation when compared to HD. Different from data reported in a previous work<sup>14</sup> where T lymphocytes and monocytes were investigated, the increase in STAT-1 phosphorylation in MS MSCs is independent on disease activity (i.e., before and after transplantation) and we did not find any correlation with transcription factors expression and production of IFN $\gamma$ , IL-10, and IL-6. We think that our data identify more a cell signature correlated with MS than a transitory marker of disease activity. Extending the observation to signal transduction factors downstream TLR4 pathway expression in monocytes/MSCs of the same sample, we showed that mRNA level for NF- $\kappa$ B, STAT-1, and I $\kappa$ B is increased in terms of relative expression and correlates with CXCL10 production. This

suggests that in our MS sample TLR4, that may be elicited by PAMPs of bacterial origin as well as by self-molecules derived from stressed cells (i.e., heat-shock proteins, HSPs<sup>15</sup> or extracellular matrix components<sup>16</sup>), is more activated in two different, for origin and function, cell lineages. Indeed, TLR4 activation may contribute to disease by different routes. First, increased activation of TLR4 may be responsible for an increased systemic inflammation with consequent effects on axon injury and disease progression, following the hypothesis of the occurrence of an immune-mediated neurodegenerative process in MS.<sup>17</sup> Second, as both T and B cells do express TLRs, increased responsiveness of TLR4 may interact as costimulatory factor, influencing T-cell regulatory response.<sup>18</sup> Third, TLR4 is present in CNS-resident cells such as astrocytes and microglia and mediates immune response during inflammation; in particular, TLR4 ligands are known to be present



**Figure 4.** Signaling protein expression profile. (A and B) STAT-1, NF- $\kappa$ B, and I $\kappa$ B expression was evaluated by real-time PCR in MSCs (A) and in peripheral monocytes (B) isolated from six MS patients (gray columns) and six HD (black columns). mRNA expression levels of STAT-1, NF- $\kappa$ B, and I $\kappa$ B were determined in basal condition (–) and upon LPS stimulation for 4 h (+LPS). mRNA expression is reported as ratio to  $\beta$ -actin (relative expression). Upon LPS stimulation, both MS MSCs (A) and MS monocytes (B) express higher levels of STAT-1, NF- $\kappa$ B, and I $\kappa$ B than HD ones ( $*P < 0.05$ ,  $**P < 0.02$ , Mann–Whitney test). (C and D) Correlation between CXCL10 production (pg/mL) and STAT-1/NF- $\kappa$ B relative expression in MS MSCs (C) and MS monocytes (D) upon LPS stimulation. Each point in graphs represents an independent sample. Linear regression between CXCL10 (pg/mL) and STAT-1/NF- $\kappa$ B relative expression is statistically significant both in MSCs and in monocytes ( $P$  value and  $r^2$  value are reported in each graph). (E) STAT-1, NF- $\kappa$ B, and I $\kappa$ B expression evaluated by real-time PCR in MS MSCs and peripheral monocytes after AHSCT and compared as fold increase with pre-AHSCT and HD values. Cells were isolated from the same MS patients before and after AHSCT. mRNA expression level of STAT-1, NF- $\kappa$ B, and I $\kappa$ B was determined in basal condition (–) and upon LPS stimulation for 4 h (+LPS). Expression was evaluated as ratio to  $\beta$ -actin. Here, we show that transcription factors expression is still significantly increased in MS compared with HD ( $*P < 0.05$ ) independently on AHSCT treatment. MSCs, mesenchymal stem cells; AHSCT, autologous hematopoietic stem cell transplantation.

in MS lesions.<sup>19</sup> Recently, increased expression of TLR3, and to a lesser extent TLR4, has been described in MS-circulating cells and it has been reported to be responsible for increased innate immune response activation along with augmented production of CXCL10.<sup>20</sup> These authors hypothesize that differential TLRs expression may modulate inflammation and may be associated with MS clinical courses. Increased expression of TLR4 and TLR2 and relative signaling pathways has been described in naïve CD4<sup>+</sup> T lymphocytes isolated from secondary progressive MS (SP MS).<sup>21</sup> These authors conclude that SP MS CD4<sup>+</sup> T lymphocytes are biologically different from HD in terms

of cell activation and this functional dysregulation is associated with a subgroup of SP MS patients characterized by a strong response to anti-inflammatory therapies. Here, we followed the evidence that altered TLR4 activation, and not altered expression (data not shown and reference [2]) in BM MSCs and circulating monocytes, and probably in CNS-resident cells, is a characteristic of a cohort of MS patients, all refractory to conventional therapies but good responders to AHSCT. The consequent augmented CXCL10 production could be one of the factors that determines, establishing an inflammatory loop, the observed disease phenotype as it has been previously reported in



two not LPS-induced models of autoimmune arthritis.<sup>22</sup> In this scenario, TLR4 should have been a possible AHSCT target in MS therapy.<sup>23</sup> On the contrary, our data show that autologous transplantation does not modify the altered TLR4 signaling pathway and consequent CXCL10 hyperproduction, detected in BM and periphery cells. On the other hand, CXCL10-producing MSCs and increased CXCL10 in plasma BM, together with OPN, were recently shown not to be associated with disease activity, probably contributing to dysregulation of T-cell survival,<sup>24,25</sup> thereby influencing the outcome of AHSCT.<sup>26</sup> Against this hypothesis, our data show that the BM-altered microenvironment does not affect the therapy's efficacy. Taken together, the present results support the idea that other mechanisms are involved in AHSCT efficacy as reconstitution of lymphocyte cell repertoire.<sup>27</sup> We suggest that when the principle MS effector arm (adaptive immunity) is affected innate immunity mechanisms lose their influence on the disease course and on the outcome of the therapy.

Finally, in our study, we confirm the partially different nature of MS MSCs compared to HD, not only for the production of CXCL10 after LPS stimulation but also for down-modulation of autologous T cells during proliferative response, that results reduced (Aldinucci pers comm 2014). In a previous work, we demonstrated that MS MSCs are able to down-modulate T-cell proliferative response during MLR and shift DCs toward less immunogenic action;<sup>2</sup> the fact that they fail to reduce proliferation of autologous T cells despite they maintain the strong immunomodulatory effects on DCs is in agreement with other author observations.<sup>28</sup> Ultimately, it has been hypothesized that CXCL10 over production might contrast possible MSCs cancer-inducing effects. Indeed, together with monocytes, neutrophils, and lymphocyte, recruitment activity CXCL10 acts as antiangiogenic chemokine and it has been shown that adipose-derived MSCs transfected with CXCL10 gene downregulate MMP2 and Bcl2, and this correlates with a strong antitumor activity.<sup>29</sup>

In conclusion, our study, although performed with a small sample, individuates a molecular signature correlated with MS patients who were all good responders to AHSCT, and adds new knowledge to AHSCT mechanisms and MS MSCs nature. In the future, it should be important to further investigate MS patients for TLR4 pathway in T and B lymphocytes and to attempt identification of possible epigenetic factors responsible for the reported increase in innate immune activation.

## Acknowledgment

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## Conflict of Interest

None declared.

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